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Design, synthesis, conformational analysis and nucleic acid hybridisation properties of thymidyl pyrrolidine-amide oligonucleotide mimics (POM)†

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Pyrranol-amide oligonucleotide mimics (POM) I were designed to be stereochemically and conformationally similar to natural nucleic acids, but with an oppositely charged, cationic backbone. Molecular modelling reveals that the lowest energy conformation of a thymidyl-POB monomer is similar to the conformation adopted by ribonucleosides. An efficient solution phase synthesis of the thymidyl POM oligomers has been developed, using both N-alkylation and acylation coupling strategies. 1H NMR spectroscopy confirmed that the highly water soluble thymidyly-dimer, T2-POM, preferentially adopts both a configuration about the pyrrolidine N-atom and an overall conformation in D2O that are very similar to a typical C3'-endo nucleotide in RNA. In addition the nucleic acid hybridisation properties of a thymidyl-pentamer, T5-POM, with an N-terminal phthalimide group were evaluated using both UV spectroscopy and surface plasmon resonance (SPR). It was found that T5-POM exhibits very high affinity for complementary ssDNA and RNA, similar to that of a T5-PNA oligomer. SPR experiments also showed that T5-POM binds with high sequence fidelity to ssDNA under near physiological conditions. In addition, it was found possible to attenuate the binding affinity of T5-POM to ssDNA and RNA by varying both the ionic strength and pH. However, the most striking feature exhibited by T5-POM is an unprecedented kinetic binding selectivity for ssRNA over DNA.

Introduction

Modified nucleic acids and oligonucleotide mimics are of considerable interest as agents for down-regulating gene expression through hybridisation either with target mRNA (antisense) or dsDNA (antigene).1−6 This approach has not only led to new therapeutic treatments, but is widely used in target validation to identify new therapeutic targets. In addition, modified oligonucleotides are of value as diagnostic and bio-analytical probes, or as tools in molecular biology.6−11 These molecules are also of fundamental importance as models to explore the structure−function relationship of nucleic acids.12,13 In particular, by studying the biophysical properties of chemically modified nucleic acids, it is argued that we can gain insight as to why DNA and RNA were selected as the ubiquitous genetic materials on this planet.14

Despite the large number of backbone modifications that have been developed, surprisingly few confer enhanced recognition properties, affinity and sequence selectivity, for DNA and RNA relative to native nucleic acids.1,15 A general strategy to enhance affinity has been to develop modified oligonucleotides that are better pre-organised by restricting the conformational freedom of the backbone.1,16 For example, sugar modifications which retain the native phosphodiester linkage, such as LNA (Fig. 1),17,18 have been used to particularly good effect.19−21 Several groups have also replaced the phosphodiester linkage in nucleic acids with alternative neutral linkages based on the premise of favourable enthalpy changes resulting from diminished electrostatic repulsion between strands.1 However even the best of all neutral linkages, for example amide 319 or 3′-N-sulfamate modified DNA,20 result in only modest increases in affinity for complementary nucleic acids. Of all the neutral mimics that have been developed, probably PNA 4, where the whole backbone is replaced by a pseudo peptide structure,21−23 is the most effective and widely used.5−10 Nevertheless PNA suffers from poor aqueous solubility and a tendency to aggregate. In addition PNA is achiral and can bind in both a parallel and antiparallel fashion with nucleic acid, which can jeopardise sequence specificity.

The introduction of positive charges into the backbone of oligonucleotide mimics is an alternative approach for increasing the affinity for complementary DNA and RNA. One of the earliest examples reported was the deoxynucleic guanidine (DNG) 5 oligomers, where the phosphodiester linkages in DNA are replaced by cationic guanidinium groups.24 This
modification was shown to vastly increase affinity for complementary ssDNA and RNA. However, it remains to be seen if such strong electrostatic attraction between oppositely charged backbones results in non-specific binding, via salt bridges, which may not necessitate base pairing and therefore may not be evident from standard UV thermal denaturation experiments.

As part of current research in our laboratory into the development of backbone modified oligonucleotides, we recently introduced the novel cationic pyrrolidine-amide oligonucleotide mimic (POM). In this paper we describe the design rationale, solution phase synthesis and conformational analysis of thymidyl T₅-POM. Finally, the nucleic acid hybridisation properties of a phthalimide capped T₅-POM are discussed in detail.

Results and discussion

Design and molecular modelling

Pyrrolidine-amide Oligonucleotide Mimics (POM) 1 (Fig. 1) are derived by replacing the furanose ring and phosphodiester backbone in DNA with a (2R,4R)-configured pyrrolidine ring and amide linkage, respectively. The pyrrolidine ring was chosen because it will be substantially protonated at physiological pH. Due to nearest neighbour interactions it is anticipated that only every other pyrrolidine will be protonated at physiological pH. Nevertheless oligomers will possess high water solubility and exhibit electrostatic attraction for complementary nucleic acids, possibly increasing affinity. In this respect it is similar to DNG. It was also envisaged that the binding affinity to DNA/RNA might be attenuated by changing the ionic strength or pH, which may be an attractive property if these molecules are to be used as probes, for example in DNA-microarray type devices. In addition there is some evidence to suggest that positively charged peptides and peptidomimetics (like POM) could bind through electrostatic attraction to phosphate groups of the cell wall, which can aid transport through the cell membranes. The amide linkage was selected to connect the pyrrolidine units, since this group has been shown to be a viable replacement for phosphodiester in the amide-linked DNA mimics. Furthermore the amide linkage should facilitate the synthesis of POM using peptide chemistry and also allow easy conjugation with peptides, in a similar fashion to DNA mimics.

The (2R,4R) stereochemistry of the pyrrolidine ring was carefully selected because this should ensure that the N-acetamido group will adopt the sterically less demanding trans relative configuration making the system a stereochemical match with native nucleic acids. Of course the pyrrolidine N-atom can invert configuration via the free amine at a rate that will depend on pH. However, X-ray crystallographic data for a protonated pyrrolidine ring 6, which is stereochemically identical and both electronically and sterically similar to the pyrrolidine ring in POM, clearly show that the N-alkyl substituent adopts the trans relative stereochemistry, at least in the crystalline state (Fig. 2). In addition the overall conformation of 6, which is described by a pseudorotation phase angle (P) of 43.7° and a maximum torsional angle (ν_max) of 40.7°, is similar to uridine 7 in the crystalline state (P = 14° and ν_max = 42°). In fact pyrrolidine 6 is only a few degrees of pseudorotation out from the preferred C3'-endo conformation of ribose in RNA which typically falls within the range P = 0 to 30°. As such this was envisaged that the pyrrolidine unit of POM would bear a closer conformational resemblance to native RNA and more conservative mimics such as amide linked DNA 3 and LNA 2 which similarly adopt a C3'-endo sugar-conformation. As a result of these critical stereochemical and conformational features POM is predicted to bind in an antiparallel fashion and differs conceptually from other oligonucleotide systems incorporating pyrrolidine units. These systems were designed as conformationally restricted analogues of PNA, unlike POM which we envisage shares little conformational or electrostatic similarity with PNA.

In order to determine the relative energies of the different conformations and conformations of the pyrrolidine ring in POM, semi-empirical quantum mechanical calculations were performed on the model thymidyl-pyrrolidine cis- and trans-dimethyl diastereoisomers 8 and 9 (Fig. 3). Each conformer, generated for every 10 degrees of pseudorotation, was first energy minimised allowing free rotation of the thymine base about the C4'-N1 bond in order to optimise the pseudo-glycosidic torsion angle (χ). The standard enthalpies of formation for each conformer were then calculated (see Fig. 3 and ESI Table 1). These reveal that, as predicted, the conformers generated for the trans-diastereoisomer 9 are on the whole lower in energy than the cis. Moreover the lowest energy trans-conformer A has a phase angle of 48° which is similar to the crystal structure of pyrrolidine 6 and also just outside that region of conformational space occupied by C3'-endo ribose typical for RNA. The trans-conformer B (P = 198°) is 0.57 kcal mol⁻¹ higher in energy and analogous to a C2'-endo deoxyribose typically highly populated in DNA structures.

Whilst these findings are encouraging and suggest the most highly populated configuration and conformation will match closely the RNA ribose structure, it should be noted these
studies do not account for intramolecular forces that may arise in longer oligomers. For example, one could envisage a possible H-bond extending from the protonated pyrrolidine N-atom to an adjacent O-atom of the amide linkage in oligomeric POM 1. Nevertheless intrastrand electrostatic repulsion should favour the formation of an extended linear structure, with trans-relative stereochemistry, and disfavour the formation of tightly folded secondary structures, which might jeopardise base pairing with complementary DNA and RNA.

**Synthesis of a thymidyl-POM dimer**

In order to investigate the conformational properties of POM, using \( ^1 \)H NMR spectroscopy, the synthesis of a thymidyl-dimer, T\(_3\)-POM, was embarked upon first. Accordingly the ester 10 (Scheme 1) was prepared from trans-4-hydroxy-L-proline and further transformed to the thymidyl-pyrrolidine 11 in an analogous fashion to the corresponding methyl ester, which was prepared earlier. Reduction of the ester 11 proceeded in 69% yield with concomitant cleavage of the benzoyl-protecting group to give the alcohol 12. Whilst the cleavage of the benzoyl group was unexpected, it was nevertheless advantageous since thymine-N3 protection was employed to ensure regioselectivity in the previous Mitsunobu reaction, and was not envisaged to be necessary for subsequent transformations. Initially we sought to change the alcohol 12 to the corresponding amine 14 via the tosylate 15 and the azide 16. The tosylate 15 was thus prepared in essentially quantitative yield, but upon heating to 80 °C with LiN\(_3\) in DMF for 16 h surprisingly none of the expected azide 16 was obtained. Instead the 6-eneamine-bridged tricyclic compound 18 was isolated as the major product. Presumably under the thermal conditions of substitution reaction the azide 16 undergoes an intramolecular 1,3-dipolar cycloaddition with the C5–C6 double bond of the thymine ring. The resulting fused triazine 17 presumably spontaneously rearranges to give 18. In order to circumvent this, the alcohol 12 was converted into the corresponding phthalimide derivative 13 under standard Mitsunobu conditions. Removal of the phthaloyl group from 13 proceeded in 92% yield on treatment with 40% aqueous methylamine, at 40 °C for 1 h, to furnish amine 14.

Accordingly a solution of the primary amine 14 in dry dichloromethane was added slowly to a 65% (w/v) solution of bromoacetic anhydride in acetonitrile at ~15 °C, which resulted in a near quantitative yield of the bromoacetamide 20. Removal of the Boc protecting group from 13 under standard conditions gave the secondary amine 19 as the TFA salt in essentially quantitative yield. Coupling bromoacetamide 20 with this amine salt 19 in the presence of Et\(_3\)N and DMF, however, gave the dinucleotide analogue 21 in a disappointingly low 58% yield. A major side product from this reaction was the primary alcohol 23. However, when this coupling was attempted using DIPEA instead of Et\(_3\)N, the yield of dimer 21 rose dramatically to 98% with no observed side products. In both cases the reactions were carried out under totally anhydrous conditions. It seems most plausible that the less sterically hindered base triethylamine can deprotonate the acidic proton on thymine (N\(_3\) pK\(_a\) = 9.7) to liberate a nucleophilic neighbouring group, which could participate in an intramolecular S\(_N\)2 displacement of the bromide to give the lactam 22. This intermediate 22 could not be isolated probably because it undergoes facile hydrolysis, either on TLC or during the aqueous workup, to give the side-product 23.

Whilst the N-alkylation approach (19 + 20 → 21) using DIPEA in DMF was excellent in terms of yield, the reaction proceeded slowly at room temperature (18 h) and would therefore not be ideal for future application in the solid-phase synthesis of POM. Dimer formation using an acylation approach was therefore also investigated (Scheme 3). Thus amine 19 was treated with tert-butyl bromoacetate to give the ester 24, which upon treatment with 20% TFA in dichloromethane gave acid 25 as a TFA salt. This acid 25 was then transformed into the pentafluorophenyl ester 26 in 85% yield using pentafluorophenyl trifluoroacetate. Acylation of primary amine 14 with the Pfp-ester 26 proceeded smoothly at room temperature in dichloromethane and after 3 h gave the protected dinucleotide analogue 21 in quantitative yield.

Accordingly the ester 10 (Scheme 1) was prepared from trans-4-hydroxy-L-proline 42 and further transformed to the thymidyl-pyrrolidine 11 in an analogous fashion to the corresponding methyl ester, which was prepared earlier. Reduction of the ester 11 proceeded in 69% yield with concomitant cleavage of the benzoyl-protecting group to give the alcohol 12. Whilst the cleavage of the benzoyl group was unexpected, it was nevertheless advantageous since thymine-N3 protection was employed to ensure regioselectivity in the previous Mitsunobu reaction, and was not envisaged to be necessary for subsequent transformations. Initially we sought to change the alcohol 12 to the corresponding amine 14 via the tosylate 15 and the azide 16. The tosylate 15 was thus prepared in essentially quantitative yield, but upon heating to 80 °C with LiN\(_3\) in DMF for 16 h surprisingly none of the expected azide 16 was obtained. Instead the 6-eneamine-bridged tricyclic compound 18 was isolated as the major product. Presumably under the thermal conditions of substitution reaction the azide 16 undergoes an intramolecular 1,3-dipolar cycloaddition with the C5–C6 double bond of the thymine ring. The resulting fused triazine 17 presumably spontaneously rearranges to give 18. In order to circumvent this, the alcohol 12 was converted into the corresponding phthalimide derivative 13 under standard Mitsunobu conditions. Removal of the phthaloyl group from 13 proceeded in 92% yield on treatment with 40% aqueous methylamine, at 40 °C for 1 h, to furnish amine 14.

Two different approaches were envisaged for chain extension of POM. In the first approach N-alkylation of the pyrrolidine 19 with bromoacetamide 20 was investigated (Scheme 2).
In order to analyse the conformation of POM in aqueous solution, the tri-cationic T3-POM 30 was subjected to extensive 2D $^1$H NMR analysis. The $^1$H NMR spectrum of 30, in D$_2$O, was fully assigned with the aid of COSY-45, TOCSY, ROESY and J-resolved experiments. Vicinal coupling constants were obtained for every proton on the trisubstituted “upper” pyrrolidine ring and these are shown in Table 1. Theoretical coupling constants were also calculated, using a generalised Karplus equation,$^{46}$ and the dihedral angles obtained for the four lowest energy conformers A, B, C and D, that were derived previously from calculations performed on the model cis- and trans-diastereoisomers 8 and 9 (Fig. 3). The experimentally observed $^1J$ values from T$_2$-POM 30 were then compared with the calculated $^1J_{HH}$ values of the model conformers (Table 1).

Noticeably the H2’–H3’ coupling constant ($^1J_{2’,3’}$) of 9.9 Hz observed for the upper ring of dimer 30 (Fig. 4) corresponds well to the calculated values for both conformers A and C where the H2’–H3’ dihedral angles ($\phi_{2’,3’}$) are 157° and 155° respectively. In contrast both conformers B and D have identical pucker ($P = 198^\circ$) and thus equivalent dihedral angles ($\phi_{2’,3’} = 92^\circ$), which correspond to a typically small calculated $^1J_{2’,3’}$ of 1.3 Hz. Similarly, the observed $^1J_{2,3}$ and $^1J_{4,5}$ coupling constants of 6.2 and 3.7 Hz correspond more closely with the calculated values for either structure A or C. Thus, the observed $^1J$ values are in best overall agreement with the structures A ($P = 48^\circ$) or C ($P = 38^\circ$), both of which are analogous to the northern or C3’-endo ribose ring conformations.

### Table 1 $^1$H NMR conformational analysis of T$_2$-POM·3HCl 30

<table>
<thead>
<tr>
<th>Vicinal H–H</th>
<th>Observed $^1J_{HH}$/Hz for 30</th>
<th>Calculated $^1J_{HH}$/Hz (dihedral angle $\phi_{HH}$/deg)$^a$</th>
<th>Karplus parameters [r]$^b$</th>
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<tr>
<td></td>
<td></td>
<td>for conformer A</td>
<td>C</td>
</tr>
<tr>
<td>2’–3’</td>
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<td>10.2$^a$</td>
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<td></td>
<td>(157)$^c$</td>
<td>(155)</td>
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<td>5.9</td>
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<td></td>
<td>(42)</td>
<td>(41)</td>
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<td>6.7</td>
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<td></td>
<td>(143)</td>
<td>(136)</td>
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<tr>
<td>3’–4’</td>
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<td></td>
<td>(25)</td>
<td>(20)</td>
</tr>
<tr>
<td>4’–5’</td>
<td>3.7</td>
<td>3.4</td>
<td>2.5</td>
</tr>
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<td>(110)</td>
</tr>
<tr>
<td>4’–5’</td>
<td>8.8</td>
<td>9.0</td>
<td>8.9</td>
</tr>
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<td></td>
<td>(1)</td>
<td>(7)</td>
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$^a$ The observed $^1J_{HH}$ coupling constants for the tri-substituted “upper” pyrrolidine ring of T$_2$-POM·3HCl 30. $^b$ The calculated $^1J_{HH}$ coupling constants and dihedral angles for the vicinal protons of the lowest energy conformers A–D of the model cis- and trans-dimethyl pyrrolidines 8 and 9 (Fig. 3). $^c$ The Karplus parameters $a_1$, $b_1$ and $c_1$ derived previously for t-4-hydroxyproline$^{50}$ were used in the generalised Karplus equation to calculate $^1J_{HH}$ (see Experimental section).

and then treated with more methylamine to give amine 27 in a combined yield of 75%. Treatment of the amine 27 with HCl gave the totally deprotected hydrochloride salt T$_2$-POM 30 that was used in the following conformational studies.

### NMR conformational analysis of T$_2$-POM

In order to analyse the conformation of POM in aqueous solution, the tri-cationic T$_2$-POM 30 was subjected to extensive 2D $^1$H NMR analysis. The $^1$H NMR spectrum of 30, in D$_2$O, was fully assigned with the aid of COSY-45, TOCSY, ROESY and J-resolved experiments. Vicinal coupling constants were obtained for every proton on the trisubstituted “upper” pyrrolidine ring and these are shown in Table 1. Theoretical coupling constants were also calculated, using a generalised Karplus equation,$^{46}$ and the dihedral angles obtained for the four lowest energy conformers A, B, C and D, that were derived previously from calculations performed on the model cis- and trans-diastereoisomers 8 and 9 (Fig. 3). The experimentally observed $^1J$ values from T$_2$-POM 30 were then compared with the calculated $^1J_{HH}$ values of the model conformers (Table 1).
where the C7’–C6’ dihedral angle is 76° which places the corresponding protons within 2.5 Å. However, in conformer B, the corresponding dihedral angle is much larger, 155°, which places these protons more than 3.8 Å apart (Fig. 4). Interestingly, H6–H3' and H6–H5' cross-peaks are also detected as well as a H6–H4' cross-peak, which suggests that the thymine ring is in equilibrium between the anti and syn conformations, relative to the pyrrolidine ring. A similar conformational equilibrium is often observed with natural nucleosides. In summary both observed JFH coupling constants and ROEs for the tri-substituted “upper” pyrrolidine ring in 30 are consistent with the conformer A (Fig. 4). This configuration and conformation was also the lowest energy structure determined by modelling. Whilst it is possible that the tri-substituted pyrrolidine ring in 30 is in equilibrium between several conformations, in solution, the fact that the NMR and modelling studies are consistent suggests that the most highly populated, lowest energy structure is that which most closely matches a typical nucleotide in RNA.

Synthesis of T₄-POM

For our preliminary investigations into the nucleic acid hybridisation properties of POM we decided to synthesise and evaluate T₄-POM. The synthesis toward T₄-POM proceeded using iterative alkylation of the pyrrolidine ring with the bromoacetamide 20 already in hand. Accordingly, Boc protected T₄-POM 21 was treated with trifluoroacetic acid to give the secondary amine 31, as the TFA salt. Coupling of 31 with bromoacetamide 20 proceeds in DMF with excess DIPEA to give Boc protected T₄-POM 32, in near quantitative yield (Scheme 4). Repeating this Boc deprotection/coupling cycle leads to the Boc protected T₄ and T₄-POM 33 and 34 respectively, in similarly satisfactory yields after purification by silica gel chromatography. Complete assignment of the 1H and 13C NMR spectra of these protected oligomers 32–34 was significantly complicated due to spectral overlap. Nevertheless the signals of the thymine CH₂ and C4 carbon atoms are clearly resolved in the 13C NMR spectra recorded in CD₂OD (see ESI Fig. 2), whilst the thymine NH signals can be distinguished from the 1H NMR in DMSO-d₆ (see ESI Fig. 3). Attempts to remove the phthaloyl group of Phth-T₄-Boc POM 34, again using aqueous methanolic, proved unsuccessful and were ultimately abandoned. It appears that base catalysed decomposition, involving attack of the N-terminal amine on the central amide linkage to give bicyclic lactam 28, is considerably more significant in the case of the pentamer 34 than the dimer 21 (Scheme 3). Thus the final step in the synthesis was achieved by treating Phth-T₄-Boc POM 34 with aqueous hydrochloric acid. Evaporation and lyophilisation gave Phth-T₄-POM 35, as its HCl salt, which was greater than 95% pure as determined by analytical reverse phase HPLC (see ESI Fig. 4). All subsequent nucleic acid binding studies were carried out with this oligomer 35, retaining the phthaloyl group. Subsequent as yet unpublished results have indicated that the N-terminal phthaloyl moiety has little effect on DNA/RNA binding properties compared with N-acetyl capped oligomers.

UV spectroscopic analysis of T₄-POM–nucleic acid binding properties

Phth-T₄-POM binding poly(rA). UV thermal denaturation experiments were performed in order to evaluate the affinity of Phth-T₄-POM 35 upon hybridisation with RNA. Initially the variation of absorption (A₅₉₀) of an equimolar mixture of Phth-T₄-POM 35 and poly(rA) (42 µM each in bases) in 10 mM K₂HPO₄ buffer adjusted to 0.12 M K⁺, pH 7.0, was recorded. A complete cycle of the thermal induced denaturation and renaturation involves fast heating (5 °C min⁻¹), slow cooling (0.2 °C min⁻¹), followed by slow heating (0.2 °C min⁻¹). The slow heating curve resulted in a single 26% hyperchromic shift with a melting temperature (Tₘ) of 48.5 °C (ca. 10 °C Tₘ/base) (Fig. 5a). Under identical conditions corresponding native d(T)₅ did not show any hyperchromic shift with poly(rA) above 8 °C, whilst the denaturation of d(T)₅ and poly(rA) has a Tₘ of 42.0 °C (ca. 2 °C Tₘ/base). A T₄-PNA containing a Lys residue at either terminus exhibits similar affinity for poly(rA), as Phth-T₄-POM 35, with a Tₘ of 56 °C. While it is often hazardous to extrapolate a Tₘ value obtained with homo-oligomers into an accurate value of Tₘ per modification found in a mixed sequence, it is clear that Phth-T₄-POM 35 binds with extremely high affinity to poly(rA). Thus it would seem that the pyrrolidine ring imposes a favourable conformation for hybridisation with RNA. Based on results from modelling and NMR studies (vide supra) this is likely to be, at least in part, a result of the favourable northern C3′-endo type conformation adopted by the pyrrolidine ring.

Interestingly, substantial hysteresis was evident between the cooling and heating curves in this experiment (see ESI Fig. 6) indicating that the rate of heating/cooling employed (0.2 °C min⁻¹) is faster than the rate of association/dissociation of Phth-T₄-POM 35 and poly(rA) such that a true equilibrium is not attained. This is in contrast to native duplex forming nucleic acids which show essentially no hysteresis at this rate of temperature change, implying Phth-T₄-POM 35 hybridises more slowly to poly(rA) than do native nucleic acids. Since it is impossible to retrieve thermodynamic data unless the heating/cooling curves are coincident, an even slower rate of heating/cooling was employed. However, even at a heating/cooling rate of 0.1 °C min⁻¹, equilibrium conditions were still not realised. Thus in order to obtain a true equilibrium Tₘ value, an equimolar sample of Phth-T₄-POM 35 and poly(rA) was subjected to a series of cooling/heating ramps performed at five different rates (5, 2, 1, 0.5 and 0.1 °C min⁻¹). It was evident from these curves that the slower rates of annealing result in higher Tₘ values and greater hyperchromic shifts (see ESI Figs. 7 and 8). By extrapolating to an infinitely slow rate of heating/cooling, the true equilibrium Tₘ was determined to be 49 °C and is 0.5 °C higher than that observed at 0.2 °C min⁻¹. All further heating/cooling experiments were performed at 0.2 °C min⁻¹ and so the quoted Tₘ values are slightly lower than the true equilibrium values and stand uncorrected.

Fidelity and stoichiometry of T₄-POM binding RNA. In order to assess the fidelity of Phth-T₄-POM 35 binding to RNA, UV denaturation experiments were carried out with 35 and the
non-complementary homopolymers poly(rG), (rC) and (U) (Fig. 5b). In all three cases no significant hyperchromicity is observed, hence it can be concluded that Phth-T₅-POM 35 does not bind to the non-complementary homopolymers by a typical base-pairing/stacking mechanism. These results do not, however, rule out non-specific binding, via electrostatic interactions between the oppositely charged backbones.

A single hyperchromic shift was observed in the thermal denaturation of Phth-T₅-POM 35 and poly(rA) which is consistent with the melting of a Watson and Crick base paired duplex or the simultaneous dissociation of the three strands of a T.A.T triple helix. In order to determine the binding stoichiometry and discriminate between these two possibilities the method of UV continuous variation was employed. Here UV absorbance, over the range 220–300 nm, was measured as a function of the molar ratio of bases, for mixtures of Phth-T₅-POM 35 and poly(rA). An incubation period of 24 h prior to measurements was found to give the clearest results, presumably due to the relatively slow rate of association between 35 and poly(rA). A Job plot of the percentage change in A₂₆₀ against mole ratio of T : A shows a well-defined minimum at 1 : 1 consistent with duplex formation. In addition, the minimum in the Job plot occurs at the intersection of two straight lines, indicating a reversibly formed complex with few or no vacant sites.

**Effect of ionic strength and pH on Tₘ for Phth-T₅-POM binding RNA.** Bruce and co-workers showed that decrease in ionic strength results in an increase in the binding affinity of the positively charged oligonucleotide DNG 5 (Fig. 1) to poly(dA) and poly(rA). This is due the “intimate” association between the more “naked” oppositely charged backbones at lower salt concentration. In order to determine whether electrostatic attraction could contribute to the stability of the duplex formed between Phth-T₅-POM 35 and poly(rA), melting experiments were carried out at different ionic strength over a ten-fold concentration range. Surprisingly, there is actually a slight increase in duplex stability with increasing ionic strength between 0.12 and 12 M K⁺ (Tₘ = 48.5 and 55.0°C respectively), which equates to an increase in Tₘ/base of ca. 1°C (Fig. 6a). This implies that electrostatic attraction is not a major contributing factor to the duplex stability. This may be a consequence of either there being a relatively large distance between the positive charge in POM and the negative charge of the phosphodiester group in a duplex with RNA, or that Phth-T₅-POM 35 is only partially protonated at pH 7.0. In addition, it may be that the observed increased affinity of 35 for poly(rA) at higher ionic strength is due to 35 adopting a slightly different conformation at higher salt concentration. It was also noticeable from the UV melting curves, recorded at different salt concentrations, that the hyperchromicity decreases significantly, whilst hysteresis between the heating and cooling curves increases, at higher ionic strengths (see ESI Fig. 9). This is probably due to slower rates of association and dissociation at higher ionic strength.

In order to assess the effect of pH on the affinity of Phth-T₅-POM 35 binding to poly(rA), melting experiments were carried out at several different pH values over the range 6–8 (10 mM
phosphate buffer adjusted to 0.12 M $K^+$). This revealed that there is a substantial increase in $T_m$ from 45 to 57°C ($\Delta T_m$/base $= 2.1$ °C) on lowering the pH from 8.0 to 6.0 (Fig. 6a). Thus, protonation of the nitrogen atom of the pyrrolidine ring is an important factor in the formation of more stable duplexes. The fact that duplex stability increases with salt concentration suggests that conformational changes brought about by protonation are more likely to be the cause of the observed increase in duplex stability at lower pH, than electrostatic attraction. From the melting curves (see ESI Fig. 10) it was again noticeable that greater hyperchromic shifts and reduced hysteresis are observed at the melting curves (see ESI Fig. 10) it was again noticeable that greater hyperchromic shifts and reduced hysteresis are observed at lower pH, suggesting that binding occurs faster at lower pH. The fact that there was significant hysteresis between the heating and cooling curves even at pH 6.0 indicates binding is still slow relative to native duplex hybridisation.

**Phth-T$_5$-POM binding DNA.** In contrast to the distinct melting curves obtained with poly(rA), repeated UV heating/cooling experiments with an equimolar mixture (42 $\mu$M each in bases) of Phth-T$_5$-POM 35 and poly(dA) revealed no hyperchromic shifts between 15 and 93 °C. Surprisingly, a modest cooperative hyperchromic shift could be detected after a five-fold increased concentration of both 35 and poly(dA) (210 $\mu$M each in bases) was incubated at 25 °C for 48 h, before the volume was readjusted to 1.0 mL (42 $\mu$M each in bases) (Fig. 7a). However, the $T_m$ of 57.0 °C (11.4 °C/base) was only just detectable from the sloping baselines, indicating only a fraction of the single strands had fully annealed. Thus Phth-T$_5$-POM 35 can hybridise with poly(rA) in the order of minutes (42 $\mu$M each in bases), but remarkably only partially hybridises with poly(dA) at a five-fold increased concentration after an extended period of 2 days incubation. It is also of note that although Phth-T$_5$-POM 35 binds extremely slowly to poly(dA), it does so with a higher affinity than for poly(rA) (increase in $T_m$ of 8 °C at 0.12 M $K^+$, pH 7). In addition, 35 binds to poly(dA) with higher affinity compared to Lys-T$_5$-Lys-PNA, which gave rise to a $T_m$ of 48 °C (9.6 °C/base). All subsequent $T_m$ values for equimolar mixtures of 35 and poly(dA) were obtained by incubating the two complementary strands at a five-fold increased concentration (210 $\mu$M each in bases) for at least 48 h prior to diluting the sample five-fold. While obtaining $T_m$ values with poly(dA) proved more difficult than with poly(rA), the trends appear the same for both series (Fig. 6b). Phth-T$_5$-POM 35 binds to poly(dA) with higher affinity at higher buffer strength and lower pH. In some cases two hyperchromic shifts could be detected, suggesting triple helix formation. However, due to the extremely slow binding kinetics, the stoichiometry of binding could not be satisfactorily determined, using the method of UV continuous variation. Indeed, although the Job plot for 35 and poly(dA), obtained at an ionic strength of 0.12 M and pH 7.0, gave a minimum at approximately 30% T indicative of a 1:1 helix, this was poorly defined.

Following our initial communication of POM, it was reported that incorporation of either one or two thymidylpyrrolidine units, with the same stereochemistry as POM (2/R,4/R), into ssDNA results in substantially destabilised duplexes and triplexes, relative to wild-type, upon hybridisation with complementary ssDNA and dsDNA, respectively. Our results using a fully modified Phth-T$_5$-POM 35 thus highlight that it is clearly very hazardous, and unadvisable, to extrapolate $T_m$ values based on such a simple chimeric approach as a means for understanding the nucleic acid binding properties of fully modified novel nucleic acid mimics.

**UV kinetic analysis of Phth-T$_5$-POM binding DNA and RNA.** Phth-T$_5$-POM binds faster to RNA than DNA. Since UV thermal denaturation experiments suggested there to be a substantial kinetic selectivity for Phth-T$_5$-POM 35 binding to poly(rA) over poly(dA), we decided to monitor the binding events as a function of time. Initially absorbance changes at 260 nm were monitored at 25 °C immediately upon mixing an equimolar amount of 35 into a buffered solution containing either poly(rA) or poly(dA) (42 $\mu$M each in bases, 0.12 M $K^+$, pH 7). Under these conditions a hyperchromic shift of 29% was observed after 200 min upon mixing 35 with poly(rA) (Fig. 7b). However when 35 was similarly mixed with poly(dA) no drop in $A_{260}$ was observed even after 16 h. It was only after increasing the concentration of each strand five-fold to 210 $\mu$M (in bases), that hybridisation between Phth-T$_5$-POM and poly(dA) can be observed. At this elevated concentration a moderate absorbance drop of only ca. 6% was observed after 200 min, remarkably $A_{260}$ was still dropping even after 12 h. These experiments clearly confirm that Phth-T$_5$-POM 35 binds much faster to RNA than DNA. Overall, however, it is also evident that binding of Phth-T$_5$-POM to poly(rA) is slow when compared with Lys-T$_5$-Lys-PNA. In an identical UV kinetic experiment PNA hybridisation with poly(rA) (42 $\mu$M each in bases) is essentially complete after 10 minutes, whilst Phth-T$_5$-
POM fails to reach equilibrium even after 100 minutes (see ESI Fig. 11).

To the best of our knowledge, there have been no reports of a kinetically selective sequence specific association of a ligand for RNA over ssDNA (or vice-versa). However other systems have been reported to display weak but thermodynamically selective binding for single stranded RNA over DNA. For example, 2',5'-linked RNA, 2',5'-linked DNA and 5'-linked thioformacetal-modified oligonucleotides all show substantial thermodynamic selectivity for hetero-duplex formation with RNA over ssDNA, albeit they bind with lower affinity to complementary RNA than do the corresponding iso-sequential DNA oligonucleotides. At present we have no satisfactory model to explain this kinetic selectivity of Phth-T₅-POM for poly(rA) over poly(dA) and further studies will be required to delineate the mechanism of binding. Perhaps binding of Phth-T₅-POM to poly(dA) induces a major conformational change to poly(dA) (or vice-versa), which retards further binding. It is noteworthy that a chiral PNA thymidyl decamer with a (2R,4R)-N-aminoethyl-t-proline backbone reported by Vilain et al., which differs only in the position of the amide group along the backbone, was also found to bind selectively to poly(rA) and not poly(dA). However, no investigation into binding kinetics was reported and it is thus tempting to postulate that longer incubation times would likewise reveal some binding to poly(dA). Ganesh and co-workers found no such selectivity with the stereoisomers (2S,4S) and (2R,4S) of this N-aminoethyl proline based oligomer. However, these oligomers were chimeras with just one pyrrolidine unit incorporated in a standard PNA strand. Thus any conclusions that were drawn from this work are, at best, fragile.

**Ionic strength and pH also affect the rates of hybridisation.** Similar UV kinetic experiments were also carried out to determine the effects of changing ionic strength and pH on the kinetics of Phth-T₅-POM binding poly(rA) (vide supra) at lower ionic strengths. This confirms that greater hyperchromic shifts observed in the UV thermal denaturation of Phth-T₅-POM 35 binding poly(rA) (see Fig. 7b and ESI Figs. 12 and 13). This reveals that binding is much faster at lower ionic strengths. This confirms that greater hyperchromic shifts observed in the UV thermal denaturation of Phth-T₅-POM 35 binding poly(rA) (see Fig. 7b and ESI Figs. 12 and 13). This reveals that binding is much faster at lower ionic strengths. This confirms that greater hyperchromic shifts observed in the UV thermal denaturation of Phth-T₅-POM 35 binding poly(rA) (see Fig. 7b and ESI Figs. 12 and 13). This reveals that binding is much faster at lower ionic strengths. This confirms that greater hyperchromic shifts observed in the UV thermal denaturation of Phth-T₅-POM 35 binding poly(rA) (see Fig. 7b and ESI Figs. 12 and 13). This reveals that binding is much faster at lower ionic strengths. This confirms that greater hyperchromic shifts observed in the UV thermal denaturation of Phth-T₅-POM 35 binding poly(rA) (see Fig. 7b and ESI Figs. 12 and 13). This reveals that binding is much faster at lower ionic strengths. This confirms that greater hyperchromic shifts observed in the UV thermal denaturation of Phth-T₅-POM 35 binding poly(rA) (see Fig. 7b and ESI Figs. 12 and 13). This reveals that binding is much faster at lower ionic strengths. This confirms that greater hyperchromic shifts observed in the UV thermal denaturation of Phth-T₅-POM 35 binding poly(rA) (see Fig. 7b and ESI Figs. 12 and 13). This reveals that binding is much faster at lower ionic strengths. This confirms that greater hyperchromic shifts observed in the UV thermal denaturation of Phth-T₅-POM 35 binding poly(rA) (see Fig. 7b and ESI Figs. 12 and 13). This reveals that binding is much faster at lower ionic strengths. This confirms that greater hyperchromic shifts observed in the UV thermal denaturation of Phth-T₅-POM 35 binding poly(rA) (see Fig. 7b and ESI Figs. 12 and 13). This reveals that binding is much faster at lower ionic strengths. This confirms that greater hyperchromic shifts observed in the UV thermal denaturation of Phth-T₅-POM 35 binding poly(rA) (see Fig. 7b and ESI Figs. 12 and 13). This reveals that binding is much faster at lower ionic strengths. This confirms that greater hyperchromic shifts observed in the UV thermal denaturation of Phth-T₅-POM 35 binding poly(rA) (see Fig. 7b and ESI Figs. 12 and 13). This reveals that binding is much faster at lower ionic strengths. This confirms that greater hyperchromic shifts observed in the UV thermal denaturation of Phth-T₅-POM 35 binding poly(rA) (see Fig. 7b and ESI Figs. 12 and 13). This reveals that binding is much faster at lower ionic strengths. This confirms that greater hyperchromic shifts observed in the UV thermal denaturation of Phth-T₅-POM 35 binding poly(rA) (see Fig. 7b and ESI Figs. 12 and 13). This reveals that binding is much faster at lower ionic strengths. This confirms...
the overall trends at pH 6.0 are very similar to those at pH 7.0, and is in agreement with the UV spectroscopic measurements. This is likely to be a consequence of enhanced electrostatic attraction between the more protonated Phth-T-POM 35 and ssDNA, presumably arising from electrostatic attraction, such that the specific Watson–Crick type base-pairing is disrupted. This also represents the first example of a modified oligonucleotide capable of switching from sequence specific to sequence non-specific binding of a nucleic acid under the control of an external stimulus (pH). Finally it is noteworthy that the rate of dissociation of Phth-T-POM 35 from the mixed sequence ssDNA (NFxB) at pH 5.0 is much faster than from d(AGC), suggesting that, in the presence of a complementary sequence, Phth-T-POM binds to d(AGC) preferentially via Watson–Crick type base-pairing.

Summary

An efficient synthetic route to the highly water-soluble cationic dimucleotide analogue T5-POM 30, has been developed. 1H NMR spectroscopy has shown that the N-acetamido group of the protonated pyrrolidine ring in POM adopts a preferred trans-configuration relative to the base and thus the backbone is stereochemically equivalent to native nucleic acids. Semi-empirical quantum mechanical calculations carried out on dimethylpyrrolidine nucleoside analogues also support this observation. In addition, it was found that the pyrrolidine ring preferentially adopts a conformation similar to the northern-type conformation adopted by ribose in RNA. Moreover, since the thymine base was found to be suitably oriented for intermolecular hydrogen bonding, thymidyl POM satisfies the necessary configurational and conformational requirements for hybridisation with DNA and RNA.

Oligonucleotide analogues comprised of a pyrrolidine-amide backbone are emerging as a new class of nucleic acid mimics with desirable properties.36,37,40 Our preliminary studies have found that the pyrrolidine-amide oligonucleotide mimics (POM), in particular, exhibit many desirable, as well as unforeseen properties. Solution-phase synthesis of the highly water soluble thymidyl pentamer Phth-T-POM was achieved using an efficient iterative N-alkylation strategy. UV spectroscopy and surface plasmon resonance have shown that Phth-T-POM binds with very high affinity and specificity to complementary ssDNA and RNA. Surprisingly, upon increasing the ionic strength, slightly elevated electrostatic interactions between oppositely charged backbones does not play a significant role in binding affinity. Decreasing the pH resulted in elevated Tm’s which is possibly a consequence of conformational changes brought about by protonation of the pyrrolidine ring. By contrast, Phth-T-POM was found to bind to complementary RNA faster at lower ionic strength and pH, implying that electrostatic interactions are important in determining the kinetics of hybridisation. SPR analysis has also shown unequivocally that Phth-T-POM maintains the fidelity of sequence specific binding (base-pairing) observed with native nucleic acids, at both pH 7.0 and 6.0. However, at pH 5.0 the cationic oligonucleotide was attracted to a non-complementary ssDNA, presumably via electrostatic contacts between oppositely charged backbones. Intriguingly, Phth-T-POM was found to bind much faster to RNA than to DNA. We are currently developing a solid-phase synthesis of longer mixed sequence POM in order to explore in greater depth this kinetically selective recognition, which may provide new insight into the conformational differences between the two native nucleic acid forms.

Experimental section

1H NMR spectra were recorded at 270, 300, 400 or 500 MHz. 13C NMR spectra were recorded at 67.9, 75.5, or 100.6 MHz. Chemical shifts are reported in parts per million relative to Me4Si or residual solvent signal. 31P NMR spectra were recorded at 202.06 MHz and referenced to 85% H3PO4 in D2O.
recorded at 188.2 MHz unreferenced. FAB mass spectra were obtained on a ZAB-SE VG Analytical Fisons Instrument. Electron Impact (EI) and Chemical Ionisation (CI) mass spectra were recorded using a VG Analytical 70 70 EQ mass spectrometer using NH₃ as carrier gas. Electrospray ionisation (ESI) mass spectra were performed, in positive ion mode, using a Q-Tof (Micromass, Manchester). Infra-red spectra were acquired on a Perkin-Elmer 783 as KBr discs or CHCl₃ solutions. Melt- ing points were determined using a Cambridge Instrumental microscope with a Reichert-Jung heating mantle and are un- corrected.

Optical rotations were measured at 20 °C with an Optical Activity AA-1000 polarimeter. Flash silica column chromatography was carried out over Merck Kieselgel 60 (230–400 mesh) and Merck Kieselgel 60 F254 0.25 mm plates were used for analytical TLC. Reactions involving anhydrous condi- tions were carried out in flame-dried glassware under a pos- itive pressure of argon. All solvents were distilled before use. Reagents were purified and solvents dried using standard procedures.

Molecular modelling

Co-ordinates of X-ray crystal structures of uridine 7⁻ and a pyrrolidine HCl salt 6²⁹ were obtained from the Cam- bridge Crystallographic Database. The pseudorotation phase angle (P) and degree of pucker or maximum torsional angle (νₑₘₓₑₚₑₜ) are calculated by substituting the published torsion angles (ν₁ → νₚₑₜ) into the equations: 

\[ P = \frac{(\nu_1 + \nu_2) - (\nu_3 + \nu_4)}{2} \cdot \sin(36^0 + \sin72^0) \]

\[ \nu_{\text{max}} = \nu_{\text{pct}} \cos P \]²⁹ Models of the cis- and trans-dimethylpyrolidines 8 and 9 (Fig. 3) were built from the co-ordinates of the reported crystal structure of pyrrolidine 6. For both diastereoisomers 35 conformations, for every 10 degrees of pseudorotation, were energy minimised, allowing only free rotation of the thymine base about the C₄’–N₁ bond, in order to optimise the C₃’–C₄’–N₁–O₂ torsion angle which is analogous to the glycosyl torsion angle in native nucleic acids (ψ)²⁹ The enthalpies of formation for each con- former were then calculated by semi-empirical quantum mechanical calculations using MOPAC 6.0 software ⁴⁴ on a Silicon Graphics workstation. The maximum torsional angle (νₑₘₓₑₚₑₜ) was optimised to 40°. The standard enthalpies of formation, phase angles (P), cyclic torsion angles ψ → ψₑₜ, and the torsion angle C₃’–C₄’–N₁–C₂ (ψ for conformers A–D) are shown in ESI (Table 1). The structures of conformers A and B are shown in Fig. 4.

NMR conformational analysis

NMR spectra of T₂-POC-3HCl 30 (ca. 4 mg) dissolved in 99.9% D₂O (0.5 ml) were recorded at 500 MHz on a Bruker DRX500 at 30 °C and referenced to the residual HDO peak at 4.70 ppm. A ¹H spectral width of 5000 Hz was used through- out. 2D TOCSY, COSY 45, ROESY and J-resolved spectra were collected using 8k data points in τ₂ and 256 τ₁ increments, typically zero-filled to 8k × 512 data points, with a relaxation delay of 2 seconds. Suppression of residual solvent signal (HDO) was achieved using presaturation during the relaxation delay.

ROESY and TOCSY spectra had typical mixing times of 300 and 60 ms respectively. The assignment of all proton chemical shifts, vicinal coupling constants, and ROESY cross peaks are given in ESI. The observed J_HH coupling constants for the tri-substituted (upper) pyrrole ring of T₂-POC-3HCl 30 (Table 1) were compared with the calculated J_HH coupling constants for the corresponding vicinal protons for the lowest energy conformers A–D of the model cis- and trans-dimethylpyrolidines 8 and 9 (Fig. 3).

The calculated J_HH coupling constants are derived from the general Karplus equa- tion \[ J = a \cos^2 \phi + b \sin \phi \cos \phi + c \phi \]²⁹ The Karplus parameters used in the calculations, a, b, and c, are listed in Table 1 and are the same as those derived for 1,4-hydroxyproline.²⁹

N-(tert-Butoxycarbonyl)-trans-4-hydroxy-proline ethyl ester, 25% eq. NH₂ (1.8 ml) was added to a solution of N-(tert-butoxycarbonyl)-trans-4-hydroxy-proline ethyl ester (3.50 g, 13.4 mmol) in CH₃OH (30 ml) and the mixture was then stirred at room temperature for 5 h. Evaporation of solvent under reduced pressure and subsequent purification by flash chromatography, using ethyl acetate as eluant, gave the title compound (2.90 ± 0.91%) as a viscous transparent oil. Rₐ 0.35 (3:1 ethyl acetate–hexane); [α]₂⁰ = +67.4 (c = 2.0, EtOH) [lit.²⁵ [α]₂⁰ = +70.4 (c = 2.0, EtOH)]; [α]₂⁰ = 300 MHz, CDCl₃ 1.28 (3H, t, J 7.2, CH₃(1′)), 1.41 and 1.46 (total 9H, 2 × C(6′)) (trans-20-27H, 1H, H₃, 2.18–2.65 (2H, CH₂(3′)), 1.35–1.70, 2.17 (1H, CH₃, 1.5) 2.20–2.29 (1H, H, H₃), 3.29–3.49 (1H, H, H₃), 3.58–3.74 (2H, H, CH₂(4′)), 4.22 (2H, J 7.0, CH₂(3′)), 4.34–4.47 (3H, 1 × t, each J 7.9, H₂ rotamers), 5.43 (1H, br s, H₄), 8.02 and 21.20 (total 1H, s, HCO rotamers); δ₋₁³C (100 MHz, CDCl₃) 28.6 and 28.7 (C(CH₃)), 35.8 and 36.9 (C₃ rotamers), 52.2 and 52.5 (C₅ rotamers), 57.9 and 58.2 (C₂ rotamers), 61.6 (CH₂(4′)), 71.1 and 72.0 (C₄ rotamers), 81.0 (C(CH₃)), 153.9 and 154.4 (CO₂Bu rotamers), 160.4 and 160.5 (HCO rotamers), 172.6 and 172.8 (CO₂Et rotamers); m/z (FAB)²⁵ 288 ([M + H]+), 317, 127, 117, 104, 93, 81, 69, 57, 45, 33, 21, 9.}

N-(tert-Butoxycarbonyl)-trans-4-hydroxy-proline ethyl ester (11). Diethyl azodicarboxylate (DEAD) (1.0 ml, 6.37 mmol) was added dropwise over 1 h to a solution of N-(tert-butoxycarbonyl)-trans-4-hydroxy-proline ethyl ester (1.50 g, 5.79 mmol) and Ph₂N (1.72 g, 6.37 mmol) in THF (50 ml) at −15 °C. The mixture was warmed to room temperature and stirred for 18 h. Evaporation under reduced pressure and purifica- tion by column chromatography, eluting with 5% acetone in CH₂Cl₂, then 5% CH₂Cl₂ in CH₂Cl₂, and finally 5% CH₂Cl₂ in CH₂Cl₂ gave 5% acetone in CH₂Cl₂, then 5% CH₂Cl₂ in CH₂Cl₂, and finally 5% CH₂Cl₂ in CH₂Cl₂ gave

\[ (300 MHz, CDCl₃) 1.28 (3H, t, J 7.2, CH₃(1′)), 1.41 and 1.46 (total 9H, 2 × C(6′)) (trans-20-27H, 1H, H₃, 2.18–2.65 (2H, CH₂(3′)), 1.35–1.70, 2.17 (1H, CH₃, 1.5) 2.20–2.29 (1H, H, H₃), 3.29–3.49 (1H, H, H₃), 3.58–3.74 (2H, H, CH₂(4′)), 4.22 (2H, J 7.0, CH₂(3′)), 4.34–4.47 (3H, 1 × t, each J 7.9, H₂ rotamers), 5.43 (1H, br s, H₄), 8.02 and 21.20 (total 1H, s, HCO rotamers); δ₋₁³C (100 MHz, CDCl₃) 28.6 and 28.7 (C(CH₃)), 35.8 and 36.9 (C₃ rotamers), 52.2 and 52.5 (C₅ rotamers), 57.9 and 58.2 (C₂ rotamers), 61.6 (CH₂(4′)), 71.1 and 72.0 (C₄ rotamers), 81.0 (C(CH₃)), 153.9 and 154.4 (CO₂Bu rotamers), 160.4 and 160.5 (HCO rotamers), 172.6 and 172.8 (CO₂Et rotamers); m/z (FAB)²⁵ 288 ([M + H]+), 317, 127, 117, 104, 93, 81, 69, 57, 45, 33, 21, 9.}
CH₃CH₂). 4.22–4.43 (1H, m, H₂), 5.18 and 5.35 (total 1H, 2 × br s, H₄' rotamers), 7.31 (1H, s, H₆), 7.42 (2H, t, J = 7.5, Br-p-H), 7.84 (2H, d, J = 7.1, Br-o-H); δₖ (100.6 MHz, CDCl₃) 12.5 and 13.0 (thymine CH₂ rotamers), 14.6 (CH₃CH₂), 28.6 (C(Ch₃)₂), 36.1 and 36.3 (C₅' rotamers), 49.9 (C₅'), 52.8 (C₄'), 57.9 (C₂'), 61.6 and 62.0 (CH₃CH₂ rotamers), 81.6 (C(Ch₃)), 121.2 (C₅), 129.1 and 129.5 (Bz-m-C rotamers), 130.8 (Bz o-C), 132.0 (Br icaco (CDCl₃) 12.5 (thymine CH₂), 28.3 (C(Ch₃)), 36.1 and 36.5 (C₃' rotamers), 51.8 (C₄'), 3.27 (1H, dd, J = 8.8, H₃), 3.05–3.20 (1H, m, H₆), 2.40–2.49 (1H, m, H₅), 28.6 (C(Ch₃)), 4.00–4.15 (2H, m, C₄H₇), 1.96–2.00 (4H, m, C₃H₇). N-(tert-Butyoxycarbonyl)-cis-4'-(thymin-1-yl)-o-prolinol (12). Lithium borohydride (49 mg, 2.24 mmol) was added portionwise in 10 mL dry THF at 0 °C. Stirring was continued at 0 °C for a further 30 min, then the mixture was allowed to warm to room temperature and stirred overnight. The reaction was cooled to 0 °C, quenched by the addition of methanol (20 mL), and evaporated under reduced pressure. The mixture was extracted with CH₂Cl₂ (2 × 20 mL), dried over anhydrous MgSO₄ and evaporated under reduced pressure. Purification by column chromatography (10% CH₂O in CH₂Cl₂) gave the enamine 18 (38 mg, 72% based on unrecovered starting material) as a white crystalline solid. §R (methanol, 4 mL) was added and the mixture was then evaporated under reduced pressure. Purification by column chromatography (10% CH₂O in CH₂Cl₂) gave the enamine 18 (38 mg, 72% based on unrecovered starting material) as a white crystalline solid. 

**RAW TEXT END**
HRMS m/z (FAB\(^{+}\)) 325.1864 (M + H\(^{+}\)), C\(_{4}\)H\(_{22}\)N\(_{2}\)O\(_{4}\) requires m/z 325.1876.

(2'R,4'R)-2'-(Phthalimidomethyl)-4-(thymin-1-yl)pyrrolidin-1-ide trithioacetic acid salt (19). CF\(_{2}\)CO\(_{2}\)H (2.0 mL) was added to a solution of phthalimide derivative 13 (830 mg, 1.83 mmol) in dichloromethane (4.0 mL). After stirring at room temperature for 4 h the solution was reduced in volume under a stream of argon to give a thick oil. On addition of diethyl ether (5.0 mL) and cooling to 0 °C, a precipitate formed which was filtered and dried to give the desired amine salt 19 (740 mg, 88%) as a white solid. \(\delta_{C} = 68.3 (100, \text{CH}_{2} \text{CO})\), 52.9 (C3), 43.5 (C4), 27.4 (C5), 21.6 (C6), 16.6 (C7). HRMS m/z (FAB\(^{+}\)) 355.2031 requires m/z 355.2030.

N-(tert-Butoxy carbonyl)-(2'R,4'R)-2'-(bromoacetoamido)-methyl-4-(thymin-1-yl)pyrrolidin-20). To a 65% (v/v) solution of bromoacetic anhydride (13 mL, 0.031 mmol) in acetonitrile was added dichloromethane (500 µL). This was cooled to -8 °C and a solution of amine 14 (10 mg, 0.031 mmol) in dichloromethane (1 mL) was added. After 5 min, the flask was warmed to room temperature and evaporated under reduced pressure. Column chromatography (5–10% CH\(_{2}\)OH in CH\(_{2}\)Cl\(_{2}\)) gave the bromoacetamide 17 (13.4 mg, 94%) as a white solid.

Phth-T\(_{2}\)-Boc-POM (21) via optimised N-alkylation. Dipea (0.33 mL, 1.89 mmol) was added to a solution of amine 19 (300 mg, 0.63 mmol) and bromoacetamide 20 (280 mg, 0.63 mmol) in dry DMF (2 mL) and the mixture stirred under argon at room temperature for 18 h. Evaporation under reduced pressure gave a residue to which was added dibenzyl ether (10 mL). The mixture was extracted with CH\(_{2}\)Cl\(_{2}\) (4 × 10 mL), dried over anhydrous MgSO\(_{4}\), filtered, evaporated and then purified by column chromatography (10% CH\(_{2}\)OH in CH\(_{2}\)Cl\(_{2}\)) to give Phth-T\(_{2}\)-Boc-POM 21 (440 mg, 98%) as a white crystalline solid.
Phth-CH₃), 3.83 (1H, d, J = 17.0, CH₂H₂CO₂Bu), 4.78–4.88 (1H, m, H4'), 7.62–7.70 and 7.71–7.79 (each 2H, m, Ar), 7.81 (1H, s, H6), 8.97 (1H, s, thymine NH); δf (67.9 MHz, CDCl₃) 12.0 (thymine CH), 28.1 (C(6)), 37.0 (C3), 39.0 (Phth-CH₂), 52.4 (C4'), 54.0 (CH₂CO₂Bu), 57.8 (C5'), 61.2 (C2'), 81.3 (C(1C1)), 110.6 (C5), 123.3, 131.8 and 134.2 (3 × Ar), 137.9 (C6), 150.9 (C2'), 163.5 (C4'), 168.4 (Phth CO) and 169.6 (CO₂Bu); m/z (FAB') 469 (M + H'), 25%); 431 (44), 314 (100), 254 (85); HRMS m/z (FAB') 469.2103 (M + H').

N-Carboxymethyl-(2'R,4'R)-2'-[phthalimidomethyl]ethyl-4'-[(thymin-1-yl)pyrrolidine trifluoroacetic acid salt (25)]. Trifluoroacetic acid (0.5 mL) was added to a solution of tert-butyl ester 24 (72 mg, 0.154 mmol) in dichloromethane (2.0 mL) and the mixture was stirred for 3 h at room temperature. Evaporation under reduced pressure followed by column chromatography (5–15% CH₂OH in CH₂Cl₂) gave the acid 25 (81 mg, 100%) as a white foam. Rf 0.62 (20% CH₂OH in CH₂Cl₂); [δ]₀₋₀.º (c = 0.5, CH₂OH): δH (300 MHz, CDCl₃) 1.90–2.10 (1H, m, H3'), 2.42–2.55 (1H, m, H3'), 2.60–2.76 (1H, m, H5'), 3.04–3.18 (1H, m, H6'), 3.45–3.51 (1H, d, J = 17.0, CH₂H₂CO₂), 3.50–3.60 (1H, m, H5'), 3.65–3.75 (1H, m, Phth-CH₃H), 3.85 (1H, dd, J = 14.7, 4.9, Phth-CH₃H), 3.96 (1H, d, J = 17.0, CO₂Bu), 4.48–4.57 (1H, m, H4'), 7.67 (4H, s, Ar), 7.95 (1H, s, H6); δF (75.5 MHz, CDCl₃) 12.5 (thymine CH₂), 37.6 (C3'), 39.6 (Phth-CH₃), 56.0 (CH₂CO₂H), 56.2 (C4'), 58.5 (C5'), 63.6 (C2'), 111.2 (C5), 117.0 (q, J = 244.1, CF₂), 124.6, 133.6 and 135.9 (Ar), 141.5 (C6), 153.4 (C2'), 163.1 (q, J = 88.6, CF₂CO₂), 166.6 (C4'), 170.6 (Phth CO), 176.7 (CO₂H); m/z (FAB') 545 ([M + Cs⁺]⁺), 435 ([M + Na⁺]⁺), 423 ([M + H⁺]⁺), 24, 392 (20), 344 (100); HRMS (FAB') 413.1443 ([M + H⁺]'), C₁₃H₁₂N₈O₅ requires m/z, 413.1461.

N-(Pentafluorophenoxycarbonyl)ethyl-(2'R,4'R)-2'-[phthalimidomethyl]ethyl-4'-[(thymin-1-yl)pyrrolidine (26)]. Dry pyridine (5 µL, 0.063 mmol) and pentafluorophenyl trifluoroacetate (6 µL, 0.035 mmol) were added to a solution of acid 25 (15 mg, 0.029 mmol) in dry DMF (100 µL). The mixture was stirred at room temperature for 2 h and then evaporated under reduced pressure. Ethyl acetate (2 mL) and 5% aqueous NaHCO₃ (2 mL) were then added and the mixture was worked up with ethyl acetate (5 × 1 mL). The extracts were dried over anhydrous MgSO₄, filtered, evaporated under reduced pressure and then purified by column chromatography (ethyl acetate) to give the ester 26 (13 mg, 78%) as a white foam. δH (300 MHz, CDCl₃) 1.17 (3H, s, thymine CH₃), 1.88–1.99 (1H, m, H3'), 2.39–2.48 (1H, m, H3'), 2.77 (1H, dd, J = 11.3, 6.8, H5'), 3.00–3.09 (1H, m, H2'), 3.30 (1H, d, J = 10.9, H6'), 3.52 (1H, d, J = 17.7, NCH₂HCO₂), 3.80 (1H, dd, J = 14.7, 1.9, Phth-CH₃H), 3.90 (1H, dd, J = 14.7, 4.5, Phth-CH₃H), 4.43 (1H, d, J = 17.7, NCH₂HCO₂), 4.83–4.92 (1H, m, H4'), 7.61 (1H, d, J = 11.4, H6), 7.68 (2H, dd, J = 5.6, 3.03, Ar), 7.77 (2H, dd, J = 5.5, 3.03, Ar), 7.95 (1H, s, thymine NH); δF (188.2 MHz, CDCl₃) 84.0 (2F, dd, J = 21.8, 12.5, 8.8, H5'), 3.51 (2H, dd, J = 13.6, 3.4, H6'), 3.70 (1H, d, J = 13.4, 7.8, H5'), 3.79 (1H, d, J = 16.1, H7'), 3.81 (1H, dd, J = 13.4, 2.8, H5'), 3.86 (2H, dd, J = 15.3, 4.1, H6' and H6*), 3.91 (1H, dd, J = 12.5, 3.7, H5'), 3.93 (1H, d, J = 11.1, 7.4, 4.1, H4*), 4.11 (1H, d, J = 16.1, H7), 4.86 (1H, dd, J = 9.6, 7.8, 6.5, 2.8, H4*), 5.03 (1H, ddd, J = 9.6, 8.8, 6.2, 3.7, H4'), 7.48 (1H, s, H6*), 7.61 (1H, s, H6).* Denotes lower pyrimidine ring (see ESI for numbering system). δC (100 MHz, CDCl₃) 23.6 and 116.7 (2 × thymine CH), 31.6 and 33.4 (2 × C3'), 39.1 and 39.3 (2 × C5'), 49.5, 55.1, 59.3 (2 × C6', NCH₂CO₂), 63.7 (C5), 69.9 (C4), 71.4 (C2'), 110.8 and 111.5 (2 × C5), 142.1 and 143.5 (2 × C6), 152.3 and 152.7 (2 × C2), 166.8 and 167.0 (2 × C4), 171.5 (CH₃CO₂H); m/z (FAB') 511 ([M – 3HCl + Na⁺]⁺), 489 ([M – 3HCl + H⁺]'), 100); HRMS (FAB') 489.2585 ([M – 3HCl + H⁺]''), C₂₁H₁₄N₈O₇ requires m/z, 489.2574.

Phth-CH₃-phosphonic acid (27). Phth-CH₃-phosphonic acid 27 (370 mg, 0.515 mmol) was added to a 20% solution of CF₂CO₂H in CH₂Cl₂ (10 mL) and stirred at room temperature for 4 h. The solvent was removed under a stream of argon followed by evaporation under reduced pressure. Dry DMF (2 mL), bromoaacetic acid 20 (229 mg, 0.515 mmol) and DIPEA (450 µL, 2.58 mmol) were then added and...
The solution was stirred at room temperature for 16 h. Evaporation under reduced pressure and purification by column chromatography (10—20% CH₂OH in CH₂Cl₂) gave Phth-T₄-Boc-POM 32 (0.490 g, 97%) as a white solid. Rf 0.27 (97% CH₂OH and 1% Et₂N in CH₂Cl₂); δₜ (300 MHz, CD₂OD) 1.21 (3H, s, thymine CH3), 1.51 (9H, s, C(CH₃)₃), 1.87 and 1.98 (each 3H, s, thymine CH3), 1.98–3.20 (12H, m), 3.30–4.40 (13H, m), 4.58–4.67, 4.67–4.82 and 4.97–5.08 (each 1H, CH₂); 5.17 (1H, s, CH6), 7.73–7.79 (4H, m, Ar), 8.07 and 8.09 (each 1H, s, H6); δc (75.5 MHz, CD₂OD) 12.5 and 13.2 (3 × thymine CH₂), 33.9, 36.5, 37.6, 39.6, 43.4, 43.5, 54.3, 55.2, 55.7, 56.6, 57.5, 57.9, 58.5, 60.2, 63.0, 63.6 and 65.3 (3 × C3, 3 × C4, 3 × C5*, 2 × C10CONH₂, Phth-CH2 and 2 × NCH₂CONH₂), 71.2 (C(CH₃)₃), 111.0, 111.8 and 112.0 (3 × C5), 127.4, 133.7, 133.5 and 135.9 (Ar C), 140.8, 141.0 and 141.1 (3 × C6), 151.3, 153.2 and 153.6 (3 × C2), 156.7 (Boc CO), 166.5, 166.7 and 167.2 (3 × C4), 170.5 (Phth CO), 173.6 and 174.2 (2 × CH₂CONH); m/z (FAB⁺) 1005 [(M + Na)+], 598 [(M + H)+], 883 (42), 466 (48); HRMS ml/z (FAB⁺) 983.4334 [(M + H)+], C₄₀H₄₀N₂O₄ requires ml/z 983.4375.

Phth-T₄-Boc-POM (33)

Phth-T₄-Boc-POM 33 (410 mg, 0.418 mmol) was added to a 20% solution of CF₂CO₂H in CH₂Cl₂ (10 mL) and the solution stirred at room temperature for 4 h. The solvent was removed under a stream of argon followed by evaporation under reduced pressure. Dry DMSO (2 mL), bromoacetamide (20 (185 mg, 0.418 mmol) and then DIPEA (440 µL, 2.53 mmol) were added and the solution stirred at room temperature for 16 h. Evaporation under reduced pressure and then purification by column chromatography (15% CH₂OH in CH₂Cl₂) gave Phth-T₄-Boc-POM 33 (500 mg, 96%) as a white solid. δₜ (400 MHz, CD₂OD) 1.08, 1.76, 1.83 and 1.85 (each 3H, s, thymine CH3), 1.38 (9H, s, C(CH₃)₃), 1.55–1.64 and 1.78–1.82 (each 1H, m, H₃), 1.87–1.95 (2H, m, 2 × H₂), 1.99–4.00 (30H, 3 × NCH₂CH₂Ph, Phth-CH2-CH₂-H₂, 4 × H₂, 4 × H₃, 4 × H5 and 3 × C10CONH₂), 4.48–4.54 (1H, m, H₄), 4.54–4.68 (1H, m, H₄), 4.85–4.93 (1H, m, H₄), 4.75–4.85 (probably 1H, H₄(m) marked by residual HDQ), 7.36, 7.87 and 7.92 and 8.00 (each 1H, CH₃), 7.64 and 7.65 (each 2H, s, thymine CH₃), 1.57, 1.65, 1.72, 1.75 and 1.78 (total 15H, 5 × thymine CH3). 2.00–4.40 (43H, 4 × NCH₂CH₂H₂*, 1 × Phth-CH2-H₂, 4 × H₂, 3 × H₅, 4 × H5 and 4 × NCH₂CONH₂), 4.38–4.67 (5H, m, 5 × H₄), 7.33–7.55 (5H, m, 5 × H₆), 7.81 and 7.82 (total 4H, 2 × s, Ar); ml/z (ESI⁻) 706.3 ([M − 3H − 5Cl]⁻), C₃₀H₂₉N₂O₂ requires ml/z 706.3 and 471.2 ([M − 2H − 5Cl]⁻), C₂₈H₂₅N₂O₂ requires ml/z 471.2.

UV hybridisation studies

General. A Varian-Cary 1 UV-Visible spectrophotometer equipped with Peltier heating block and six cell transport mechanism was employed. Quartz cuvettes (24 × 5 × 10 mm) were fitted with Teflon stoppers were used throughout. Reference cuvettes always contained the same buffer used in the corresponding sample cuvette. Data were analysed using software provided by Varian-Cary. For thermal denaturation experiments, absorbance readings were recorded at 260 nm (unless stated otherwise) with data collection every 0.1 °C and an averaging time of 1 s. Temperature monitoring was via a probe placed inside a cuvette containing buffer and adjacent to the sample cuvette in the cell holder. Concentrations of all oligonucleotides were expressed in mol/base and for each of the oligonucleotides the concentration was 42 pmol in bases, unless stated otherwise. The concentrations of stock solutions were obtained via serial dilution and for polynucleotides and d(T)₃₀ these were determined spectrophotometrically at 80 °C using the Beer–Lambert law and the known extinction coefficients of the corresponding nucleotides [δ/dm³ mol⁻¹ cm⁻¹] 15000 for dA, 15000 for RA, 7600 for rC, 12160 for rG, 10320 for rU and 8500 for dT]. For Phth-T₄-Boc-POM 35, the extinction coefficient used was 8920 which represents the sum of the extinction coefficients of thymine (ε = 8500) plus 1/5 of the extinction coefficient of pthalimide (ε = 2100) at 260 nm. All stock solutions were stored at −20 °C in between use. All sample vials and pipette tips were sterile and the H₂O used was doubly distilled prior to use. Silicon oil (Sigma) was used to prevent sample evaporation. All polynucleotides were commercially available (Sigma) while the 5-mer Lys-T₄-LysNH₂-PNA (N-terminal lysine and C-terminal lysine amide) was purchased from PE/Appplied Biosystems, Warrington, UK.

Sample preparation. Unless stated otherwise, thermal melt experiments were carried out using equimolar amounts (in bases) of both oligonucleotides taken from aqueous stock solutions and adding 500 µL of double concentrated buffer. Water was then added to give a total volume of 1 mL and the solution then mixed gently using a pipette before adding it to the cuvette. A thin layer of oil was then added and a Teflon stopper was inserted to prevent evaporation.

† Probably two signals are coincident.

‡ Two peaks are coincident.
Procedures for thermal denaturation experiments. For thermal denaturation experiments involving Phth-T$_3$-POM 35 and poly(rA), an equimolar mixture of the two strands (42 µM each in bases) was initially heated at 5 °C min$^{-1}$ from 25–93 °C in order to completely dissociate the strands. After 1 min at 93 °C, the sample was cooled at 0.2 °C min$^{-1}$ to 15 °C and then after a further 1 min the sample was heated at 0.2 °C min$^{-1}$ to 93 °C. For thermal melting experiments involving Phth-T$_3$-POM 35 and poly(dA), an equimolar mixture of the two strands was incubated at a concentration of 210 µM each (in bases) at 25 °C for at least 48 h. The sample was then diluted with the appropriate buffer to 1 mL to give a concentration of 42 µM each (in bases). After cooling from 25–15 °C at 1 °C min$^{-1}$, the sample was allowed to equilibrate at 15 °C for at least 1 min and then heated and cooled between 15 and 93 °C at 0.2 °C min$^{-1}$. For thermal melting experiments involving d(T)$_{12}$ or Lys-T$_3$-LysNH$_2$-PNA with either poly(rA) or poly(dA), a protocol identical to that used for the Phth-T$_3$-POM 35-poly(rA) thermal melting experiments was followed. For all $T_m$ determination, the temperature at the maximum of the first derivative of the slow melting curve was used. All melting experiments were performed at least twice (except where stated otherwise) and the values shown are the average of these readings.

Procedures for continuous variation experiments. In order to determine the stoichiometry for Phth-T$_3$-POM 35 binding to poly(rA), different mole ratios of the two strands at 10% increments and a total base concentration of 50 µM in 10 mM K$_2$HPO$_4$ buffer adjusted to 0.12 M K$^+$ and pH 7.0, were mixed by pipette and then allowed to anneal for 24 h at 25 °C. The mixtures were then sequentially transferred into a cuvette and the UV spectra recorded between 300 and 220 nm.

Procedures for kinetic experiments. Using the Varian-Cary kinetic software, absorbance changes at 260 nm were monitored immediately upon adding and mixing an equimolar amount of either Phth-T$_3$-POM 35 or Lys-T$_3$-LysNH$_2$-PNA to a buffer solution containing either poly(rA) or poly(dA). Experiments involving the hybridisation between Phth-T$_3$-POM 35 and poly(rA) were conducted using equimolar mixtures of each strand at base concentrations of either 42 µM or 210 µM (as poly(dA) were conducted using equimolar mixtures of each strand at base concentrations of 50 µM in 10 mM K$_2$HPO$_4$ and adjusting the time of injection to zero. Data from kinetic experiments were prepared for analysis and presentation by subtracting the average response recorded 20 s prior to injection and adjusting the time of injection to zero. Data from flow cell 1 (underivatised dextran) were subtracted from the corresponding data obtained from the flow cells (2, 3 and 4) that contained biotinylated oligonucleotides, in order to correct for bulk refractive index changes and signal drift. All oligonucleotides were biotinylated at the 5’-end using multi biotin-III-CE phosphoramidite and were purchased from Cruachem, Glasgow, UK.

Immobilisation of biotinylated oligonucleotides. Onto the second flow cell of a dextran chip containing streptavidin was injected a solution of ssDNA 5’-biotin-d(A)$_{20}$ at 1 µg mL$^{-1}$ in water for 3 min and at 10 µL min$^{-1}$ until 1450 RU were obtained. In a similar manner, flow cell 3 was derivatised with 1400 RU of ssRNA 5’-biotin-r(A)$_{20}$ and flow cell 4 with 1000 RU of ssDNA 5’-biotin-d(AGC TTC AGA GAT CGA TCG GAG AGA GTA CTG). After at least 100 s of buffer wash, 10 µL of 10 mM hydrochloric acid were passed over all the cells.

T$_3$-POM, T$_3$-PNA, d(T)$_{12}$ and d(T)$_{20}$—oligonucleotide binding assays. Serial two-fold dilutions (160–10 µM, 100 µL, 20 µL min$^{-1}$) of Phth-T$_3$-POM 35 in 10 mM K$_2$HPO$_4$ adjusted to 0.12 M K$^+$ and pH 7.0, were passed serially over all four flow cells of the sensor chip. The complex was then washed with buffer for 6 min followed by regeneration using 10 mM hydrochloric acid (20 µL, 20 µL min$^{-1}$). d(T)$_{12}$ was assayed in an identical manner, using the same concentrations. PNA (Lys-T$_3$-LysNH$_2$) was assayed in an identical manner except using serial two-fold dilutions of 40–1.25 µM and d(T)$_{20}$ using 5–0.31 µM dilutions. Assays were performed at different ionic strength and pH (as indicated) following an identical protocol.

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